

transduced. Ten years after the last cell infusion, real-time PCR analysis for the presence of vector sequences in these patients revealed that ~18% of peripheral blood mononuclear cells (PBMCs) from patient 1 carried the vector, whereas <0.1% of LASN-marked PBMCs were detectable in the second patient. Although no serious adverse events have been observed in either patient, the recent events of insertional oncogenesis in X-linked severe combined immunodeficiency patients treated with retroviral-mediated gene transfer prompted us to study the integration sites in lymphocytes from our patients with the aim of mapping the regions involved by the retroviral integration events, determining their localization with respect to known genes, and assessing whether recurrent patterns could be identified. Genomic DNA was prepared from stored PBMCs samples dating 1991, 1992, 1995, and 1996 and from 9 lymphocytic single cell clones established in 1998. Using the "inverse PCR" technique (Nolta et al. PNAS 1996; 93: 2414) we isolated ~105 integration sites from bulk PBMC samples and ~20 integration sites from single cell clones. To date, 34 of these sequences have subjected to detailed analysis. We used MegaBLAST (<http://www.ncbi.nlm.nih.gov/blast/megablast.html>) to compare the sequences to the Nov 2002 (build 31) assembly of the human genome and identified eleven sequences that mapped to a unique position in the human genome. The positions of 15,000 known genes have been determined by aligning mRNA Reference Sequences to the human genome (genome.ucsc.edu). We determined whether any of the eleven integration sites occurred within 10 kb of one of these known genes. We found one sequence within an intron of the androgen induced protein (AIG-1) gene, one sequence within an intron of the ubiquitin-conjugating enzyme E2L 3 (UBE2L3) gene, and one sequence 9 kb upstream of interleukin 1 receptor antagonist (IL1RN) gene. The remaining sequences mapped >10 kb from known genes or did not return significant similarities. Overall, our results indicate that this technique can be used to map retroviral integration sites both from bulk PBMC populations as well as single cell clones. In addition, this preliminary analysis would suggest that the integration events of the LASN vector largely did not occur within the coding regions of genes. Finally, one integration site isolated from the 1996 bulk PBMC sample was recovered also from two of the single cell clones generated 2 years later, thus demonstrating that this approach can be applied to tracking and longevity studies.

4. In Vivo Model To Assess Biosafety: Testing the Risk of RCR and Insertional Mutagenesis from Retroviral and Lentiviral Vectors

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Due to a heightened awareness of the potential for adverse events during human gene therapy trials, it is important to even more carefully document the safety of the vectors and to examine the potential for insertional mutagenesis from integrating vectors, using long-term in vivo systems combined with molecular analyses. We describe a system to assess the potential for generation of replication-competent retrovirus (RCR) and insertional mutagenesis in vivo, in immune deficient mice, followed by PCR and inverse PCR to examine integration sites. Human hematopoietic stem cells and human mesenchymal stem cells carrying two different Moloney-based vectors were co-transplanted together into immune deficient mice that are unable to reject cells that become transformed. The sensitivity of the system was initially verified using N2, a vector

with known potential to generate recombinants (Donahue et al., 1992). The N2 vector does not have the safety modifications now implemented in the LN-based vectors and packaging systems currently used in clinical trials (Lynch and Miller, 1991). Murine lymph node tumors arose in eleven of the twelve mice that received the N2-transduced human cells by IV transplantation. The neo gene was transferred to murine cells, as well as being present in human cells in each mouse, and caused a significant lymphoproliferation in the murine pre-T cells. It took five months for the leukemia to arise in those mice, and then they became extremely sick rapidly, with five of the twelve mice moribund at the same timepoint, and the others moribund within a one month period. Next, a total of 481 mice were monitored for adverse events from safety-modified vectors for 7-18 months post-transplantation. Mice that displayed any sign of ill health were killed, autopsied, and subjected to a full range of biosafety studies. No evidence of insertional mutagenesis causing human leukemia or solid tumors in any mouse was detected. No RCR were detected from the safety-modified retroviral vectors in 117 serum samples analyzed by vector rescue assay. In addition, 61 mice transplanted with human progenitors that had been transduced with HIV-1-based lentiviral vectors, and followed for 2-6 months, were assessed using similar techniques. No adverse events were caused by the vectors and no mouse tested had HIV p24 protein in their serum. The current studies provide a sensitive in vivo system to assess potential risk from replication competent vectors and from insertional mutagenesis when retroviral or lentiviral vectors are considered as candidates for human gene therapy.

5. Testing for Replication Competent Lentivirus Associated with HIV1 Lentiviral Vectors

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A principal concern regarding the safety of HIV1 based lentiviral vectors is replication competent lentivirus (RCL). We have developed two PCR assays for detecting RCL. The first assay detects recombination between the transfer vector and the packaging construct (psi-gag) and for a positive control we utilized pHIV-GFP which contains the intact psi-gag sequence. Analysis of PCR products by probing with a radiolabeled oligo revealed the sensitivity of detection of psi-gag to be ~ 10-100 copies of HIV-GFP in a background of 0.1µg of uninfected DNA (three independent experiments). The second assay we developed uses real time PCR to analyze vesicular stomatitis virus glycoprotein (VSVG) envelope DNA and has a detection limit of ~5-50 VSVG sequences in a background of 0.1µg of uninfected DNA. In an attempt to further increase the sensitivity of our detection assays, we sought to first amplify RCL on a permissive cell line – a step which will be dependent on the infectability of the cell line and the rate of RCL production. To address infectivity, we utilized a variety of cell lines (C8166, Sup-T1, CEM-SS, H9, 293 and HeLa-T4) to titer a stock VSVG pseudotyped lentiviral vector, RRL-CMV-GFP. Of the six cell lines tested, highest titer was obtained in the C8166 and 293 cell lines (2.9 x 10⁶ TU/ml, and 1.3 x 10⁶ TU/ml respectively). We next evaluated the ability of two cell lines (C8166, HeLa-T4) to amplify an attenuated HIV1 virus, R7-GFP. p24 was measured every two days for a week and a ~100 fold increase was observed by day six (300pg/ml) for C8166 where as HeLa-T4 had a minimal increase. After selecting C8166 as the amplification cell line, we compared the sensitivity of PCR and p24 ELISA. C8166 cells were infected with R7-GFP at tenfold dilutions (between 10⁻⁴ to 10⁻⁷) and each culture was analyzed by psi-gag PCR and P24 ELISA after two weeks. At the 10⁻⁴ dilution, 6/6 replicates were positive by PCR and ELISA, while 3/6 and 1/6 replicates were positive at the 10⁻⁵ dilution

for psi-gag and p24 respectively. In subsequent experiments, C8166 and 293 cells were transduced with vectors and screened for RCL weekly for 3 weeks. Psi-gag recombinants were routinely detected in cells transduced with the 4-plasmid based RRL-CMV-GFP vectors (4/4) but only occasionally in safety modified vectors that lack homology between transfer and packaging plasmid (Genetix, Inc). p24 levels above baseline were also observed (3/6), especially when testing concentrated vector. VSV-G sequences were not routinely detected. Cells exposed to media collected after 3 weeks of amplification had no evidence of psi-gag, VSVG or p24 antigen transfer, indicating that the signal detected in the amplification cells did not represent a true RCL. In conclusion, we have developed a sensitive method for RCL detection and demonstrate transfer of packaging sequence to transduced cells. To date, RCL has not been detected.

6. A High Titer, "Supersplit" Packaging System in Combination with a Self-Inactivating (SIN) Vector for the Prevention of Replication Competent Lentivirus (RCL) Contamination

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The development of a safe lentiviral packaging system able to generate high titer virus free of contaminating RCL is essential for future gene therapy trials. One of the most critical safety measures for the prevention of RCL and pre-RCL contamination is the splitting of GAGPOL. We and others (Wu et al., EMBO, 1997) have reported split packaging systems in which the GAGPOL is dissociated onto two separate plasmids. One plasmid encodes the GAG polyprotein and PR. This plasmid expresses a POL-less GAG protein, which localizes to the budding virions through myristilation of its N-terminal amino acid. The second plasmid expresses a VPR-RT-IN fusion protein, which is tethered to the budding virions through the interaction of VPR with P6. However, viral titers obtained by the split GAGPOL system were 10 fold lower than packaging systems in which the GAGPOL was not split. We hypothesized that the decrease in viral titers might be caused by suboptimal polyprotein processing by PR, possibly due to steric hindrance of PR by GAG upon separation from RT-IN. To investigate this possibility, we generated S³⁵ labeled recombinant lentivirus using split and non-split packaging systems and analyzed their protein composition. In contrast to virus generated with the non-split packaging system, virus generated with the split packaging system showed high levels of unprocessed RT-IN polyprotein strongly suggestive of suboptimal protease activity. To determine whether PR activity, and therefore viral titers, could be rescued, we further split GAG-PR onto two separate plasmids. One plasmid encodes the GAG polyprotein in which the frameshift for POL and the 207bp overlap with PR has been eliminated. The second plasmid expresses a VPR-PR fusion protein, which like the VPR-RT-IN fusion protein, is tethered to the budding virions through the interaction of VPR with P6. Analysis of S³⁵ labeled viral particles showed more efficient processing of GAG and VPR-RT-IN when PR is supplied in trans as VPR-PR fusion protein. Importantly, titer analysis of the various packaging systems showed that supersplitting PR away from GAG greatly increased viral titers to levels 3 fold lower than those obtained with the non-split packaging systems. Next, to increase safety and determine whether viral titers could be rescued further, a 400bp fragment was deleted from the U3 region of the right LTR, and the U5 region was completely removed and replaced by an strong polyA/

termination signal. These modifications resulted in a further 2 fold increase in viral titers. To demonstrate the efficacy of our "supersplit" packaging system with the SIN β 87-globin lentiviral vector, human cord blood cells were transduced, and then transplanted into NOD/SCID mice. At 24 weeks post transplantation globin was quantified by HPLC. The total h β -globin expression was comprised of 57% h β 87-globin. Here we demonstrate that the use of safety modifications such as the supersplit packaging system combined with a SIN vector can greatly increase safety of lentiviral vector systems without a loss in viral titers or efficacy.

7. When a Self Inactivating (SIN) Vector Is Not a SIN: Characterization of Episomal HIV-1 Vector Forms

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The fact that relatively large quantities of episomal vector forms are generated during retroviral vector transduction prompted us to investigate the mechanism by which non-integrating HIV-1 vector structures are formed, and to identify potential biosafety risks which may be posed by these episomal vector DNA structures, shortly after transduction. As a first step in this study, we have developed a shuttle HIV-1 vector which allows clonal isolation of circular HIV-1 DNA forms in bacteria. Using the shuttle HIV-1 vector, we were able to characterize the effect of HIV-1 integrase on the formation of circular vector DNA structures. Isolated circular vector forms were then used to develop a novel single LTR HIV-1 vector, which reduces the likelihood of recombination and rearrangements between the vectors' LTRs. We developed a variety of single-LTR HIV-1 vector constructs, which allowed production of high titer vector particles through transient three-plasmid transfection. Following integration, these vectors containing either a CMV or a tetracycline inducible promoter allowed high levels of constitutive or regulated transgene expression, respectively. More importantly, using integrase-mutant containing vector particles, we could show that episomal vector forms generated during transduction of packaging cells served as efficient templates for vector particle production. To our surprise, deletion of the U3 region from a single LTR vector construct did not reduce vector titers following three-plasmid transfection, thus indicating that single-LTR vector structures generated during transduction with traditional self-inactivating vectors, can serve as templates for vector production. To test this hypothesis, a SIN lentiviral vector was used to transduce HIV-1 packaging cells. Two days post-transduction, vector particles collected from conditioned media showed a titer of up to 105 IU/ml. Interestingly, dilution of the non-integrated vector forms during four weeks of culture resulted in a reduction of vector titers to less than 102 IU/ml. This observation indicated that the ability of SIN vectors to serve as efficient templates for vector production is restricted to their episomal forms, rather than their integrated genomes, which are not lost during cell division. To better characterize the mechanism by which full-length vector RNA is transcribed from episomal SIN vectors, 5' RACE analysis was carried on RNA extracted from SIN and Non-SIN vector-particles. The result of this analysis revealed that the 5' end of RNA molecules transcribed from a circular single-LTR SIN construct started 70-75n upstream of the beginning of the R region.

In conclusion:

A) Single-LTR HIV-1 vector constructs allow efficient production of high titer vector particles.

B) This study opens new avenues in retroviral vector design, which improve vector biosafety and broaden the spectrum of HIV-1 vector applications.

C) More importantly this study demonstrated that circular U3 deleted vectors, which are formed during transduction should not be regarded as self-inactivating.